



## Supplementary File S1: Evolving Context of Microbiota of Mammalian Milks

### A.1. Background for Benefit-Risk Assessment

As discussed in the body of this manuscript as well as Coleman and colleagues [1] and Dietert and Dietert [2], outdated dogma from 20<sup>th</sup> century science is an inappropriate basis for policies and regulations for raw milks that clearly are associated with both benefits and risks. The evidence maps for the bovine milk ecosystem generated herein (Figure 1, body of this manuscript) and the breastmilk ecosystem in a companion article [3] illustrate the major shifts in methods, concepts, and knowledge base [4] that microbiologist and physician Martin Blaser [5] described as the ‘microbiome revolution’.

Wider deliberation of the evidence of benefits and risks, the ‘state of the science’, and remaining uncertainties structured into evidence maps appears crucial for developing shared understandings of technologic advances in methodology and practice for microbial benefit-risk analysis of raw milks. Until such efforts are undertaken, outdated notions about the microbiota of milks and germophobia will likely deter development of evidence-based policies for raw and pasteurized milks.

The evidence maps generated in this project are anticipated to form the basis of an international workshop that will address the changing paradigm of milk ecosystems and initiate the first cycle of analysis and deliberation with decision makers and stakeholders in the 21<sup>st</sup> century.

### A.2. Epidemiology as a Component of Benefit-Risk Assessment

Even at the turn of the 21<sup>st</sup> century, a common belief among both scientists and regulators was that mammalian milks were sterile until contaminated by environmental microbes. Opinions about risks exceeding benefits for raw mammalian milks [6–8] appear to be oversimplifications based primarily on epidemiology and the belief that potential presence of pathogens in raw milks render it innately dangerous foods. Notably, these opinions are not informed by advances in knowledge of the natural microbiota of mammalian milks. Much of the current evidence on risks for bovine milk (Figure 1, body of this manuscript) focused narrowly on outbreaks of acute foodborne infections associated with raw milk and the rare prevalence of pathogens in routine raw milk monitoring programs worldwide ( $\leq 0.1\%$ ; Table 1, body of this manuscript). The greater societal benefits (and risks) associated with raw milks may be for chronic diseases, notably asthma and allergenicity [9]. Importantly, much of the evidence for raw milk risks is correlative, not causal.

The early development of quantitative microbial risk assessment (QMRA) in the 1990s [1,10,11] was framed on the epidemiologic disease triangle, influenced by variability in the host, the pathogen, and the environment, and interactions. However, epidemiologic studies represent only one component of multiple scientific disciplines required for QMRA and benefit-risk assessment. QMRAs estimate risk with attendant uncertainty, due to the need to rely on assumptions, weak or indirect evidence, poorly designed studies, or studies representing only partial knowledge of the causal factors leading to health and disease [1].

Two key elements of QMRAs [3] are Exposure Assessment (including predictive microbiology) and Dose-Response Assessment (including medical microbiology), both including aspects of microbial ecology of foods and the human gut. The body of evidence for factors influencing the microbiota of milks related to the ‘environment’ aspect of the traditional ‘disease triangle’ (e.g., air quality and pollution; diet; supplements and pharmaceuticals; behavior/lifestyle/environment including farm and non-farm environments, built and natural environments, organic and industrial dairy practices; dust and soil; water) is extensive and relevant to modeling dose-response relationships for pathogens amidst the dense and diverse microbiota of raw foods including milks [12].

### A.3. Methodology for 21<sup>st</sup> Century Microbial Ecology

The ‘microbiome revolution’[5] fueled tremendous expansion of knowledge of the natural microbiota of mammary tissues and mammalian milks in the past decade. Currently, milks from healthy mammals are recognized as complex living foods that contain many specialized bioactive components that function by influencing rates of growth, development and maturation of multiple mammalian tissue systems (gut, immune, neural, and respiratory) and overall health (growth factors; hormones; enzymes; cytokines and other immunologic factors; and various antibacterial compounds). A key component of the bioactivity of milks is the interdependent networks or consortia of microbial communities making up the microbiota of milks and their multifunctional effects and redundancies in metabolism of nutrients, immunomodulation, and colonization resistance against enteropathogens across microbial taxa [13–16].

The microbiota of milks are dense and diverse, containing some core microbes that typically predominate in both human breastmilk and dairy milks (*Staphylococcus*, *Streptococcus*, *Pseudomonas*, *Bifidobacterium*, *Propionibacterium*, *Bacteroides*, *Corynebacterium*, and *Enterococcus*), as well as many unique microbes present at very low abundance [15]. Cabrera-Rubio and colleagues [17] documented up to 700 bacterial species in raw breastmilk from one lactating woman. A previous systematic review on breastmilk microbiota [18] concluded that two genera are universally present in breastmilk, *Staphylococcus* and *Streptococcus*. The more recent review by Zimmerman and Curtis [19] reported higher bacterial diversity in breastmilk than in maternal or infant feces, including 58 phyla, 1,300 species, and 3,563 strains or operational taxonomic units. Other recent reviews [20,21] describe homologies between human and bovine milk in composition and function. Clearly, major functions of mammalian milks include both ‘seeding’ the gut with diverse commensal microbes and ‘feeding’ both microbial and host cells in the gut [13,22].

Estimated daily intakes of the milk microbiota for infants consuming ~800 mL of breastmilk from healthy mothers ranged from 2–8 million bacterial cells from culture-based methods ( $2.62 \log_{10}$  cfu/mL to  $\sim 10^4$  bacteria/mL)[23,24] and 80 billion bacterial cells by culture independent methods [25]. Underestimation of the diversity and abundance of bacteria in milks by traditional culture-based methods of the 20<sup>th</sup> century is likely, attributed to the limitations of knowledge for culturing most microbes naturally present in milks and the adherence of bacteria to the extracellular matrix of host cells rather than freely suspended (planktonic) bacteria in liquid portions of milk.

Limitations are also acknowledged for culture-independent methods, particularly the common tendencies to report only abundance estimates to Phylum, Family or Genus levels and the lack of standardization, as well as underestimating the influences of small sample sizes and diverse DNA preparation and reporting methods on making inferences and comparisons [16]. For example, overestimation by culture-independent methods is likely to occur based on uncertainty about viability by molecular methods used to date, as well as potential contamination by extracellular DNA or phages [25]. Despite great differences between estimated daily doses from culture-based and culture independent methods, offspring clearly ingest large numbers of microbes in raw milks each day.

Similar magnitudes of bacterial densities ( $>10^4$  cfu/mL) are reported for raw bovine milks from multiple studies [16]. These researchers compiled nearly 3,000 microbial sequence results on the microbiota of dairy products in a publicly accessible database (FoodMicrobionet; <http://www.foodmicrobionet.org/>) and reported nearly 2,000 taxa identified at the genus level or higher in raw bulk tank milk across five recent bovine microbiota studies [16]. Many of the studies report the top 10 to 50 most prevalent genera amongst hundreds of genera present in the milk microbiota to summarize in tables and figures, including genera these researchers noted as potentially beneficial microbes (*Lactobacillus*, *Streptococcus*, *Lactococcus*, *Staphylococcus*, *Corynebacterium*; [16]. However, many genera are present at densities much less than 1% total abundance [15,16]. One recent study [26] documented effects of the recommended refrigeration temperature for foods (4°C, 40°F) on abundance and diversity of cold-tolerant pseudomonads and lactic acid bacteria (LAB) in the retail raw milk microbiota.

Few milk microbiota studies are powered to identify microbes other than predominant taxa to the genus and species level by culture-independent methods, and rarely do available studies identify and quantify typical foodborne pathogens (e.g., *Campylobacter*, enteropathogenic *E. coli* strains, *Listeria*, *Salmonella*) in raw milks. Although this limitation also applied to the milk microbiome study of Liu [26] as well, the authors did analyze 16 bovine retail raw milk samples in triplicate for pathogens by culture methods and found none positive. If present, potential pathogens appear to represent extremely small fractions of the microbes present in milks from healthy individuals. In contrast, milk microbiota for individuals with mastitis, inflammation of the mammary glands, are often dominated by ‘blooms’ of opportunistic pathogens and lower diversity of microbes, suggesting that mastitis may reflect dysbiosis or disruption of the microbiota of healthy mammary tissue [15,16,23,27,28]. Note that mastitis is the most frequent disease reported in dairy cows [29]. The USDA [30] reported clinical mastitis in nearly 25% of cows sampled in 2013, and less than 5% of mastitic cows died from mammary infections. Similar rates of 20–25% mastitic infections are reported in studies of lactating women in multiple countries [31].

#### A.4. Homologies Between Human and Bovine Milk Microbiota

Oikonomou and colleagues [15] cited a systematic review on the breastmilk microbiota [18] and provided an overview of the milk microbiota in a variety of mammals including humans and cows. Oikonomou and colleagues [15] noted common taxa predominating human and bovine milks (*Staphylococcus*, *Streptococcus*, *Pseudomonas*, *Bifidobacterium*, *Propionibacterium*, *Bacteriodes*, *Corynebacterium*, and *Enterococcus*). Although the specific composition of microbiomes of human and bovine milks vary within and between individuals, populations, and studies, a systematic review on breastmilk microbiota published four years ago documents two genera as universally predominate in breastmilk: *Staphylococcus* and *Streptococcus* [18], both bacteria that metabolize lactose. Recent reviews [15,20] note similarities in human and bovine milks, including domination by these two genera in mammalian milks in the latter review.

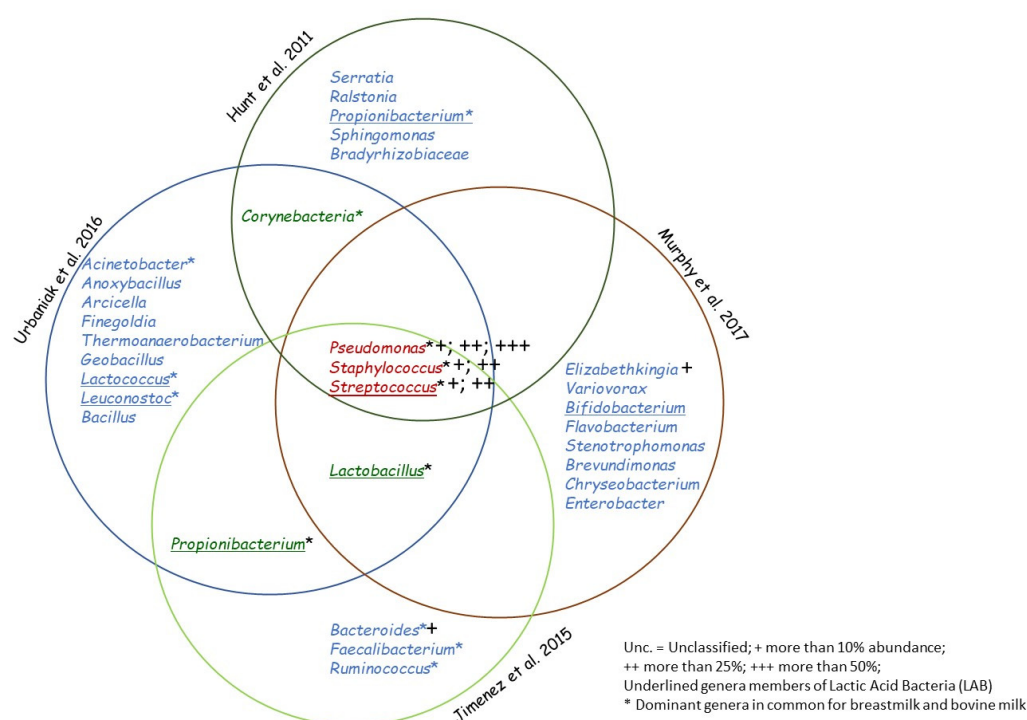
Homologies are noted for the main building blocks of milk common to all mammals, including many commonalities for milks from humans and cows [14–16,18,20,32]. These reviews note some functional similarities and differences for human and bovine milks. For example, qualitative and quantitative differences exist in oligosaccharide components of human and bovine milks that function as prebiotics (nutrients for the gut microbiota rather than directly for metabolism by the offspring). Although human milk contains higher concentrations of oligosaccharides generally of greater structural complexity than bovine milk [33], recent studies demonstrated beneficial effects of bovine milk oligosaccharides (BOS) relevant to humans: Jakobsen and colleagues [34] demonstrated BOS stimulation of a key species of *Bifidobacterium* using a simulated infant GI model system; and Kuntz and colleagues [35] demonstrated dose-dependent and breed-dependent effects of BOS in normal differentiated and transformed human intestinal cell cultures. The microbiota of both human and bovine milks generally includes members of LAB as predominant taxa of importance to health and disease. The LAB group includes diverse genera that share common metabolic and physiological characteristics: Gram positive rods and cocci; aerobic or facultative anaerobic metabolism; production of organic acids that reduce pH, decrease pathogen survival and growth, and increase shelf life of many foods; and production of antibacterial compounds including bacteriocins. These characteristics render LABs of great value as starter cultures for fermented and functional foods (e.g., yogurt and kefir), as well as human and animal supplements (probiotics) intended to promote health and protect against pathogens [36,37]. Further, a recent 12-week study documented statistically significant increases in Lactobacilli in the human gut with increased exposure to organic raw dairy products from grass-fed (pastured) cows [38].

Some LAB strains are classified as ‘Generally Recognized as Safe’ (GRAS) in foods and feeds. LABs and other bacteria in the microbiota of milks can function as probiotics, microbes benefiting human and animal gut [39] and immune

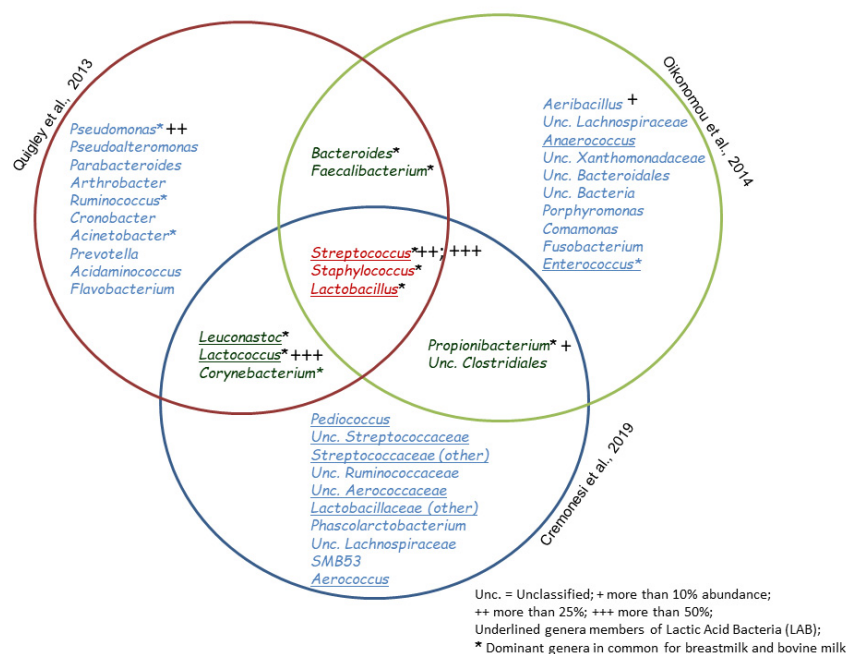
systems [40], and inducing multiple mechanisms of direct and indirect protection against enteric pathogens [20,22,39,41–46].

One recent review by Ojo-Okunola and colleagues [24] used a Venn diagram that specified the unique genera and those shared across breastmilk microbiota studies for the predominant bacterial genera. Figure A1a is a Venn diagram that was redrawn and adapted from Ojo-Okunola [24] for an additional human breastmilk study, and Figure A1b is a similar diagram prepared for this project including three bovine milk studies. Where data were available for individual animals or ranges, the + symbol in the legend for Figure A1 indicate maximal percent abundance for at least one individual and study. These Venn diagrams are consistent with the findings of the Oikonomou review [15] regarding common genera in human and bovine milk, specifically those that metabolize lactose, the major carbohydrate source in mammalian milks (*Staphylococcus* spp., *Propionibacterium* spp., and various LAB including *Bifidobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*).

**Figure A1a.** Dominant bacterial genera for breastmilk ecosystem. Redrawn and adapted from Ojo-Okunola et al., 2018 [24].



**Figure A1b.** Dominant bacterial genera for bovine milk ecosystem (Quigley, et al, 2013 [47]; Oikonomou et al. 2014 [48]; Cremonesi et al. 2019 [49])



### A.5. Microbiota of Milks: Fecal Contaminants?

Extensive literature exists documenting a plethora of interdependent factors from the micro scale to the global scale that influence the microbiota of milks (Table A1). Many of the studies cited herein are reviews or systematic reviews [15,16,18,19] that illustrate the breadth of evidence (some studies consistent, some ambiguous, some conflicting) and

the need for further deliberation about evidence for benefits and risks posed by both raw and pasteurized milks. The 20<sup>th</sup> century notion that the microbiota are simply fecal contaminants posing high risk to human health currently appears invalid. Outdated perceptions of bacteria as germs to be eradicated are gradually being replaced by deeper awareness of symbiotic (commensal and mutualistic) microbiota as our partners in health [22,50].

<b>Table A1.</b> Major Factors Influencing Composition of Mammalian Milk Microbiota	
1	genetics of the host and partnering microbes
2	health, lactation stage, and immune status of lactating mother or cow
3	birthing process (e.g., delivery mode, pre- vs full-term)
4	maternal diet including probiotics and functional foods, malnutrition
5	antibiotics and pharmaceuticals
6	milk components (immune cells; host defensins and enzymes; antibiotics produced by competing microbes; nutrients, particularly polyamines and fatty acids; vitamins and minerals)
7	microbial ecology, particularly symbiotic relationships (antagonism, mutualism) and competition for nutrients, vitamins, and minerals limiting growth in mixed populations
8	geography and environment, including air, bedding, soil, and water
9	lifestyle and/or farming conditions
10	sampling and analysis methods
11	social factors, including contacts in urban and rural environments, in family or herd environments, farming communities and cooperatives
12	economic conditions and access to health or veterinary care
13	genetics of the host and partnering microbes

Multiple lines of evidence support the plausible existence of an entero-mammary pathway for transfer of microbes or their DNA from the maternal GI tract to mammary tissue and subsequently to milk and the oral cavity and GI tract of breastfeeding infants [19,51]. The review by Oikonomou and colleagues [15] cites some of this evidence and one study [52] supporting the existence of a homologous entero-mammary pathway in bovines and concludes that the body of evidence suggests transfer of microbes from milk to infants via an entero-mammary route.

Results of the small but elegant study conducted by Wu and colleagues [53] documented the three most prevalent taxa (*Aerococcaceae*, *Staphylococcaceae*, and *Ruminococcaceae* at one farm and *Staphylococcaceae*, *Lactobacillaceae*, and *Ruminococcaceae* at another farm were shared between milk and airborne dust microbiota. Further, the milk microbiota was associated with the bedding microbiota but clearly separated from feed, rumen fluid, feces, and water microbiota. This study challenges the common assumption that bovine feces are the source of the milk microbiota.

Wu and colleagues [53] concluded that the raw bovine milk microbiota is clearly separated from the fecal microbiota (as well as the microbiota associated with feed, rumen fluid, and water). This finding contradicts the common notion that bacteria present in milk are fecal contaminants. Together with studies supporting the entero-mammary pathway of transfer of microbes in healthy hosts, these results challenge 20<sup>th</sup>-century notions about the milk microbiota and merit further deliberation for evidence-based decision making. Clearly, systematic research studies are needed to determine how generalizable these results are to other dairy farms, breeds, farm management systems including pasture-based herds, and other factors influencing the microbiota of milks.

#### A.6. Future Directions

The past decade of research characterizing the microbiota of milks documents radical advances in knowledge for these indisputably dynamic and complex ecosystems of mammary glands and milks, fueled by evolution of methodologies

for culture-independent analyses. These methods expanded knowledge not just of the dense and diverse microbiota present in milks, but more importantly, of their likely functions in the complex milk ecosystems and in healthy humans and cows. Specifically, interactions of milk microbiota are now generally accepted as essential to development and maintenance of healthy GI, respiratory, genito-urinary, and brain and nervous systems of mammalian offspring [20,22]. While the reviews cited herein provide extensive evidence characterizing the predominant microbes in milks in health and disease and the factors influencing the composition and abundance of the milk microbiota, considerable gaps exist in understanding mechanisms and predicting functionality and interdependencies of the key microbial networks in milks that benefit host systems and/or restore health for dysbiotic systems [15,20,22,54].

Microbiota researchers continue to explore interactions between microbiota and anatomical niches within mammalian bodies, including multi-directional effects along the gut-lung axis [55–60], the gut-brain axis [38], and the placenta-gut-lung triangle [61] that may further advance mechanistic understanding of benefits and risks of the microbiota of milks to human health. Future deliberations about the evidence for benefits and risks of the milk microbiota is the strength of evidence mapping applications likely to improve re-assessments and enhance the validity of statistical inferences about balancing benefits and risks while acknowledging uncertainties about likelihood and magnitude for both.

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